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Comparison of Extraction of Valuable Compounds from Microalgae by Atmospheric Pressure Plasmas and Pulsed Electric Fields

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ABSTRACT: Microalgae have recently gained interest, not only as source for biofuel, but also as a resource for pharmaceutical and nutritional substances. The bottleneck for extracting these valuable compounds from microalgae is a thick cell wall of high physical and chemical strength. Several extraction techniques are available, but suffer from different disadvantages. Therefore, new technologies are needed, especially those based on processes that will not affect the chemical composition of ingredients. Among these, physical plasma and pulsed electric fields (PEF) might be promising. Three different standard methods, microwave, ultrasound, and homogenization, were compared with plasma treatment and PEF. The plasma sources investigated were corona discharges, a plasma jet, a dielectric barrier discharge, spark discharges, and pin-to-liquid discharges. *Chlorella vulgaris* was chosen as a model organism. To detect successful cell wall rupture, the protein content of the supernatant and pigment concentration after treatment were determined. Scanning electron microscope images were taken to visualize cell wall damage. Microwave and spark discharge treatment were the most successful methods with comparable extracted total protein content in the supernatant. However, spark discharges achieved higher pigment yield than microwave extraction without the thermal degradation of the pigments observed for microwave extraction.

KEY WORDS: extraction, plasma, pulsed electric field, spark discharge, microwave, microalgae

I. INTRODUCTION

Microalgae are ubiquitously occurring photosynthetic microorganisms that can be found in freshwater and marine systems and are known for their rich content of lipids, proteins, polysaccharides, and carotenoids.[1,2] Cultivation is possible for marginal economical costs and on any scale. Microalgae can be grown in fresh water and in brackish or saline water, resulting in a low land and water footprint.[3] *Chlorella vulgaris* is one of

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the most well-characterized microalga and was first isolated by Beijernick in the 1890s.[4] For centuries, these algae have been used in the Far East as alternative medicine and are known as a traditional food source in the Orient.[5] Many proteins and carotenoids have been identified in microalgae,[2,6] in particular, high-value compounds with antitumor and immune-stimulating properties. Polysaccharides such as β-1,3-glucan are linked to antiviral, antitumor, and immune protective activities. This polysaccharide is also known for its positive influence on blood glucose level in human body. Carotenoids in microalgae, such as lutein or zeaxanthin, act as free-radical scavengers and may neutralize radicals such as hydroxyl (OH) radicals, which are related to the development of neurodegenerative and cardiovascular diseases.[7]

A major obstacle for extracting compounds from microalgae is their strong and rigid cell wall, which is composed of complex carbohydrates and glycoproteins with a high physical and chemical strength.[8] Conventional extraction processes are often energy intensive and most suffer further from the use of environmentally harmful solvents and/or long treatment times and high energy demand.[9] Moreover, changes and modifications to extracted compounds are often inevitable due to the use of these chemicals or the need for high temperatures, resulting in a loss of bioactivity or, worse, their destruction. An economically viable technique for down-streaming these processes has yet to be found.

Various physical methods based on fast heating or mechanical damage have already been used for disintegration of plant material; these include microwave exposure, ultrasound (also known as sonication), and homogenization. Amarni and Kadi showed that the extraction of oil from olives with microwaves was highly efficient. The underlying mechanism was explained by an internal heating of the *in situ* water within the biological cell. The heating accelerates cell wall rupture by sudden temperature rise. This allows a fast dissolution of the oil, which is released from broken cells into the solvent.[10] Ultrasound also showed good efficiency for cell wall disruption of plant material due to cavitation phenomena. Wu et al. used ultrasound to enhance the yield of ginseng saponins compared with Soxhlet extraction. Remarkably, this enhancement was achieved with a threefold faster treatment time compared with the classical extraction method.[11] Nevertheless, for microalgae, especially *C. vulgaris*, ultrasound does not seem to be very effective and the combination with other mechanical techniques is thus recommended.[12]

Another method for cell wall rupture, homogenization, exploits the formation of shear forces, when a biological suspension is forced through a narrow tube under high pressure. The classical method, used in microbiology for cell disintegration, is only effective for a minority of different microalgae. Additionally, in large volumes, the method consumes high amounts of energy, which makes it costly.[8] In comparison, ball milling showed that high protein yields can be achieved with moderate expenditures of treatment energy.[13]

In addition to physical methods, chemical procedures are available to disintegrate the cell wall of microalgae. For the enzymatic lysis technique, enzymes such as snailase, lysozyme, or cellulase are added to the biological sample and hydrolyze cell walls with-

out altering compounds. Zheng et al. demonstrated that lipid extraction of *C. vulgaris* could be increased up to 22–24% when lysozyme or cellulase was added. They further suggested that enzymatic lysis can be seen as a gentle and selective method. However, long treatment times of 10 h and continuously stable temperatures are required for sufficient cell wall rupture.[14] Therefore, this method may be gentle, but is also highly time and energy consuming and therefore economically unsustainable.

Nonconventional physical methods such as pulsed electric fields (PEF) or high-voltage electrical discharges have shown to enhance extraction yield from microalgae. In general, these techniques were used together with solvents and, in conjunction, could decrease the use of solvents or treatment time.[15–19]

PEF of sufficient strength and duration cause charging of cell membranes, which eventually can lead to a transient increase of permeability across membranes or a loss of membrane integrity altogether. This process of reversible or irreversible electroporation is used for incorporating large molecules such as DNA or drugs into a cell, but it can be also used to increase the extraction yield of compounds from cells.[20–22]

Plasma may also provide mechanisms that are promising to break the strong and rigid cell wall of microalgae. Depending on method of plasma generation, different processes can be exploited. Generated in water or algal suspensions, corona discharges and spark discharges provide strong electric fields, shockwaves, ultraviolet radiation, and chemical reactive species such as the hydroxyl radical, peroxynitrite, or H_2O_2.[23] In gases, plasmas can be generated in different configurations such as dielectric barrier discharges (DBDs) or plasma jets.[24,25] Depending on the method used, high amounts of reactive oxygen and reactive nitrogen species are produced with air as the working gas. In contact with or immersed in aqueous solutions,short- and long-lived species are also formed in reactions with water.[26–28] The strong oxidizing potential of the created species can be expected to at least partially dissolve the cell walls of microalgae.

Bousetta et al. showed that the extraction of polyphenols from grape seeds was enhanced using pulsed arcs. In their experiments, they compared PEF, streamers, and pulsed arcs applied directly in water. Pulses of 0.8 μs were adjusted to provide an approximate pulse energy of 3–10 J/pulse. In configurations for PEF, field strengths of 20 and 40 kV/cm were applied. Pulse repetition rates varied for the three investigated methods. Pulsed arcs in a point-to-plane configuration (gap distance, 1–3 cm) were applied with a frequency of only 2 Hz because, at higher repetition rates, a strong increase in temperature was observed. In contrast, streamers (point-to-plane gap distance, 3 cm) and PEF (plane-to-plane gap distance, 1 cm) were administered with 20 Hz. It could be shown that, compared with the other methods, pulsed arcs required between 27 and 57 times less energy to extract the polyphenols from the seeds.[16] Similar results were reported by Rajha et al., who investigated the effects of PEF, high-voltage electrical discharges, and ultrasound for the extraction of polyphenol and proteins from vine shoots. PEF of about 13 kV/cm and pulse length of 10 μs, applied at a frequency of 0.5 Hz, were investigated. The same frequency and a voltage of 40 kV were set for high-voltage electrical discharge treatment. The plasma was generated in a needle-to-plate system. A

frequency of 20 kHz was stated for ultrasound treatment. Most efficient was the treatment with high-voltage electrical discharges, resulting in the highest extracted polyphenol content with the lowest energy use.[18]

These results are encouraging for the use of plasmas for the extraction of compounds from microalgae, which has so far not been investigated. The objective of the work presented here was therefore to determine whether plasma sources are a suitable method for this purpose and which plasma source is the most successful at achieving cell disintegration of *C. vulgaris*. Therefore, corona discharges generated in the algae suspension, a plasma jet submerged into the suspension, a DBD applied above the treatment volume, and spark discharges inside the treatment volume were investigated. The different plasma sources and methods provide different direct and indirect reaction mechanisms, including different reaction chemistries and production processes of reactive species. A suitable reference method had to be found to allow a comparison and microwave extraction, ultrasound (sonication), and high pressure homogenization were studied. In addition, PEF were also investigated for the experience from biomass processing. A protein assay was established to determine protein concentration in the supernatant associated with successful cell wall rupture. This assay does not distinguish between proteins from cell wall alone; it also detects proteins from inside the cell. Furthermore, pigments were extracted and their concentration was measured photometrically after cell disruption with the respective chosen method. An increase of these compounds could indicate successful cell disintegration. In addition, scanning electron microscope (SEM) images were taken for each treatment method. Together with the extraction yields, a preliminary evaluation of energy efficiencies was also conducted for the laboratory systems.

II. MATERIALS AND METHODS

A. Cell Culture

C. vulgaris (SAG 211-12, Experimental Phycology and Culture Collection of Algae, University of Goettingen, Germany) was grown in Bold's basal modified medium (Sigma-Aldrich, Germany), containing (in mg/L): boric acid 11.42, calcium chloride dihydrate 25.0, cobalt nitrate * $6H_2O$ 0.49, cupric sulfate * $5H_2O$ 1.57, EDTA (free acid) 50.0, ferrous sulfate * $7H_2O$ 4.98, magnesium sulfate * $7H_2O$ 75.0, manganese chloride * $4H_2O$ 1.44, molybdenum trioxide 0.71, nickel chloride * $6H_2O$ 0.003, potassium hydroxide 31.0, potassium hydroxide 31.0, potassium phosphate monobasic 175.0, potassium phosphate dibasic 75.0, sodium chloride 25.0, sodium nitrate 250.0, sodium selenite 0.002, stannic chloride 0.001, vanadium sulfate * $3H_2O$ 0.0022, and zinc sulfate * $7H_2O$ 8.82.

Cells were cultured according to Schulze et al. photoautotrophically in ventilated, 500 mL Schott flasks, bubbled with air with a flow rate of approximately 1.5 vvm. Temperature was kept at 20 ± 2°C and the culture was illuminated in light:dark cycles (12:12 h) with white fluorescence lamps (50 μmol photons $m^{-2}s^{-1}$).[29]

B. Sample Preparation

For experiments, cells were harvested after inoculation between day 30 and day 45 with an adjusted optical density (OD) of 0.8 at 750 nm (Shimadzu UV-1280 spectrophotometer) for a final volume of 50 mL. Subsequently, the algae suspension was centrifuged at 3000 × *g* (centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Germany) for 10 min, washed with 20 mL of deionized water, and centrifuged at 3000 × *g* for 5 more min. The cells were kept on ice until use.

Dry weight was determined with 20 mL algae suspension (OD 0.8) in a circulating air drying oven (Heraeus Oven UT12, ThermoScientific, Germany). Experiments were repeated in triplicate.

C. Standard Methods

To determine protein yield in the supernatant and for the extraction of pigments, the algae suspensions were treated as described below with standard methods, five plasma sources, and PEF treatment, respectively. After these initial treatments, proteins and pigments were extracted as described under the "Measurement of protein content" and "Pigment extraction" sections.

1. Microwave Extraction

After washing and centrifugation, the supernatant was discarded and the cells were refilled with 3 mL deionized water, transferred into 4 mL brown-glass vials (Agilent Technologies, Germany), and closed. The cap was prepared with a septum, which allowed pressure balance. Subsequently, the vial was placed inside the microwave (Bosch, distributed by Carl Roth, Germany) and extraction was conducted at 600 W for 20 s. Afterward, the suspension was allowed to cool down to ambient temperature, refilled to 50 mL, and centrifuged for further investigations.

2. Ultrasound Extraction (Sonication)

The cell suspension was prepared as described above and filled with deionized water to a final volume of 50 mL. To avoid thermal heating due to cavitation, the extraction was conducted under ice cooling with a treatment time of 20 min and a frequency of 20 kHz (Sonopuls HD 2070 Bandelin, Germany). Subsequently, algae were centrifuged for further investigations.

3. Homogenization

After preparing the cell suspension, the liquid with a volume of 50 mL was transferred into the homogenization apparatus (EmulsiFlex-C5, Avestin, Canada). The pressure was adjusted to 5000 psi and treatment time was 8 min. One minute of treatment time ac-

counts for one cycle; therefore, the algae were pressurized in eight cycles. Afterward, the cells were prepared for further investigations as described above.

D. Plasma and PEF Treatment

1. Corona Discharges

For corona discharge treatment, a coaxial electrode geometry was used that is described in more detail by Banaschik et al.[30,31] Two pure, uncoated, twisted tungsten wires of 0.05 mm diameter (W-005135/13, Goodfellow, Huntingdon, UK) were fixed in the middle of a glass tube as inner electrodes. The twisted wires were chosen for a more durable electrode configuration during the application of discharges. A stainless steel mesh, fixed on the inner wall of the tube, served as theouter electrode. The glass tube had a length of 70 mm and an inner diameter of 34 mm, thus containing a total volume of 64 mL. High-voltage pulses were generated with a 300 ns stacked Blumlein line pulse generator with a repetition rate of 11 Hz. Voltage was measured with a 120 kV/80 MHz high-voltage probe (PMV-5, NorthStar Marana, AZ) and current with a current monitor (Model 5046, Pearson Electronics, Palo Alto, CA). Electrical parameters were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY).

The suspension was moved in a continuous flow system with a peristaltic pump (FH100x, Thermo Scientific, Waltham, MA) to avoid settling of algae in the system. In addition, the suspension was chilled during the whole treatment time to prevent temperature increase. Algae were prepared as described above and, after centrifugation, the pellet was diluted to 140 mL to fill the entire system properly. To generate long streamers, a conductivity of 70 μS/cm was necessary, which was adjusted with sodium chloride. A voltage of 80 kV was applied and treatment time was set to 30 min. After treatment, the solution was centrifuged and the cell pellet was prepared for pigment extraction. Samples from the supernatant were taken for protein measurements.

2. DC-Plasma Jet

Whether algal cell walls are affected by a plasma jet exposure was determined using a DC-plasma jet as described by Kolb et al. and Kredl et al.[32–34] In this setup, plasma is ignited within a microhollow cathode geometry. The inner electrode is made of brass and the outer electrode consists of alumina. Plasma is generated between the inner and outer electrode when a positive voltage of 2 kV is applied to the discharge circuit by a DC high-voltage power supply (PS FX06R50, Glassman High Voltage Inc., High Bridge, NJ, USA). For experiments, the discharge current was adjusted to 30 mA and the treatment time was set to 30 min. Compressed air was used as the operating gas, flowing through the inner electrode with a flow rate of 4 standard litre per minute (slm). A total of 50 mL of algae suspension with OD_{750} = 0.8 was treated. During the treatment, the algal suspension was chilled on ice.

3. Volume DBD (VDBD)

To observe cell wall rupture caused by indirect plasma treatment, a DBD plasma was applied as described by Koban et al.[35] The VDBD consisted of two metal electrodes, a Petri dish (55 × 14.2 mm, VWR International, Germany), and a titanium disc, which was placed between them. The bottom of the Petri dish was used as dielectric for the DBD. The distance between electrode and dish was 15 mm. The entire system was sealed against entry of ambient air and cooled with a Peltier element. Argon was used as the feed gas with a flow rate of 500 standard cubic centimetres per minute (sccm). Total treatment time was set to 10 min with an input voltage of 10 kV and a frequency of 33.8 kHz (AG Series Amplifier Type AG1021, T&C Power Conversion Inc., NY, USA). Forward power was set to 27 W and reflected power to 18 W. Cells were prepared as described above and the algae pellet diluted to 1 mL with deionized water. After treatment, the cell pellet was refilled with distilled water to its start volume of 50 mL for further investigations.

4. Pin-to-Liquid Discharge

As a further plasma source for the generation of reactive species, a pin-to-liquid discharge setup similar to that described by Chen et al. was used for algae treatment.[36] After centrifugation, the algae pellet was diluted to a total volume of 90 mL; otherwise, the gap between the liquid and the needle electrode would have been too large for the given setup and no plasma could have been ignited. The solution was treated with an AC-driven pin-to-liquid electrode configuration placed on top of a beaker and treatment time was set to 3 min. The electrode configuration consisted of two stainless steel electrodes. An AC transformer (F.A.R.T. 5000/75Pe), controlled by a variac (RFT LTS 002), charged the electrodes. One electrode was submerged into the liquid and the other was positioned about 3 mm above the surface of the solution. Due to considerable heating from the sparks, a treatment time longer than 3 min was not feasible. Temperatures more than 25°C were achieved within 3 min and a longer treatment time would have destroyed the algae near the surface. To ensure convection and a uniform distribution of plasma-generated species, the liquid was stirred with a magnetic stirrer (VMS-C4, VWR, Germany). Temperature was controlled with a handheld infrared thermometer (PeakTech 4975, PeakTech Prüf- und Messtechnik GmbH, Germany) pointed at the liquid surface in a 30 s cycle. After the treatment, the liquid was centrifuged and prepared for further measurements.

5. Spark Discharges

For spark discharge treatment, a chamber with a rod-to-rod-configuration was constructed. The cylindrical body of the chamber consisted of polymethyl-methacrylate and an outlet at the bottom and top of the chamber (Fig. 1). Outlets were connected with plastic tubes to a peristaltic pump (Meredos TL, Medorex, Germany). This continuous flow sys-

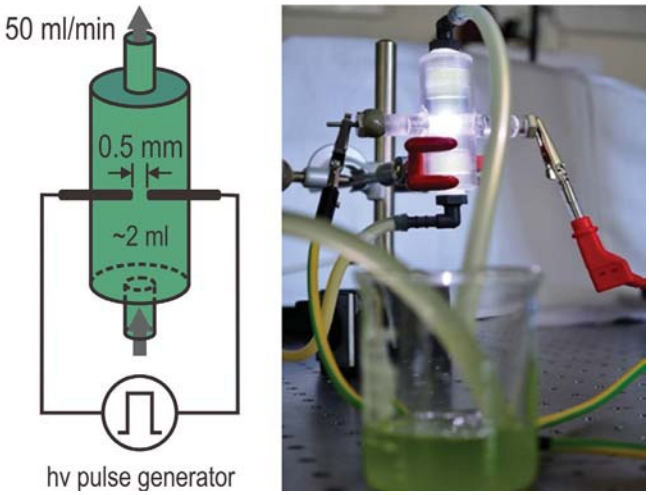

FIG. 1: Spark discharge chamber (left: schematic, right: setup) for the recirculating treatment of microalgae suspensions.

tem ensures homogenous treatment and avoids settling of algae at the bottom of the treatment chamber. The whole system, including tubes and expansion tank, held a total volume of 50 mL with the spark-discharge reactor accommodating approximately 2 mL. The flow rate was adjusted to 50 mL/min to ensure a treatment of the complete suspension in 1 min. Rectangular to the direction of suspension flow, two tungsten electrodes with a diameter of 2.4 mm and gap distance of 0.5 mm were set inside the chamber. Positive high voltage pulses were generated with a Blumlein line generator, delivering high-voltage pulses of 100 ns duration at a spark repetition rate of 4 Hz for an input voltage of about 6–7 kV from a DC high-voltage power supply (PS/EQ060R020-22, Glassman High Voltage Inc., High Bridge, NJ, USA). With the low conductivity of the algal suspension, the overall electrical resistance of the filled treatment chamber was much higher than the impedance of the pulse generator of 400 Ω. By refraining to match pulse generator and reactor impedance, a train of 100 ns high-voltage pulses with decreasing amplitudes was applied between the electrodes, with the first pulse reaching an amplitude of about 80 kV (instead of only a single pulse of only 40 kV amplitude).[37] A typical waveform is displayed in Fig. 2. The repetitively applied high electric fields eventually result in a breakdown, usually between the fourth and eighth applied pulse. The remaining energy, which is stored in the pulse generator, is then dissipated in the instigated spark discharge. The short pulse duration corresponds to a maximum energy of only 1.6 J.

Voltage was measured with a 40 kV/75 MHz high voltage probe (Tektronix P6015A, Tektronix Inc., Beaverton, OR) and current with a Pearson current monitor (Model 5046, Pearson electronics, Palo Alto, CA). Both measurements were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). Experiments were conducted within a treatment time of 30 min and temperature was controlled with

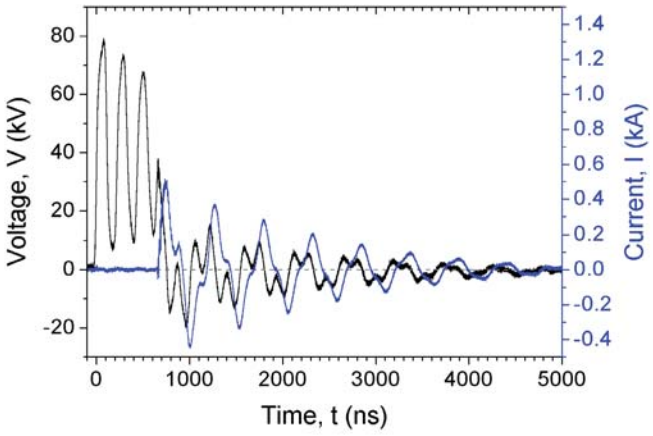

FIG. 2: Current and voltage for the spark discharge treatment. Because the resistance of the treatment chamber filled with microalgae is not matched to the impedance of the Blumlein pulse-forming network, several reflected pulses are applied across the discharge gap before a breakdown occurs and a spark is initiated.

a hand-held infrared thermometer (PeakTech 4975, PeakTech Prüf- und Messtechnik GmbH, Germany) pointed at the expansion reservoir and spark discharge chamber. For each experiment, 50 mL of algae suspension with OD_{750} = 0.8 was prepared as described in the "Sample preparation" section.

6. PEF Treatment

For PEF treatment, the algae suspension was treated in a polytetrafluoroethylene (PTFE) treatment chamber as described by Goettel et al. and Eing et al.[17,38] The same continuous-flow system with a flow rate of 50 mL/min as described for spark discharges was used to avoid settling of algae and to allow air bubbles to exit the chamber. Two stainless steel electrodes with a diameter of 32 mm were paired in parallel and separated by a gap distance of 4 mm in the PTFE housing. The system was connected to a 1 µs pulse-forming network (PFN) that consisted of 16 capacitors (paired in parallel) with 2 nF, a spark gap switch, and an impedance of 480 kΩ. The PFN delivers square pulses with a flat amplitude and a rise time of 0.13 µs into a matched load. One thousand pulses with an amplitude of the first unmatched pulse of 14 kV were administered (resulting in a field strength of 35 kV/cm) at a frequency of 5.5 Hz (power supply Glassman High Voltage Inc., High Bridge, NJ, USA). Voltage was measured with a 40 kV/75 MHz high voltage probe (Tektronix P6015A, Tektronix Inc., Beaverton, OR) and current was measured with a Pearson monitor (Model 5046, Pearson Electronics, Palo Alto, CA). These parameters were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). Because the pulse generator impedance and load impedance of the treatment chamber were not the same (matched), a train of pulses with decreasing amplitude

similar to the system described in the "Spark discharges" section were applied without any breakdown. A typical voltage waveform is shown in Fig. 3.

All exposure parameters were adapted from Goettel et al. and Eing et al.[17,38] For all other experiments, 50 mL of algae suspension with OD_{750} = 0.8 was prepared as described above and treated.

All investigated methods with their essential treatment parameters are described in Tables 1 and 2.

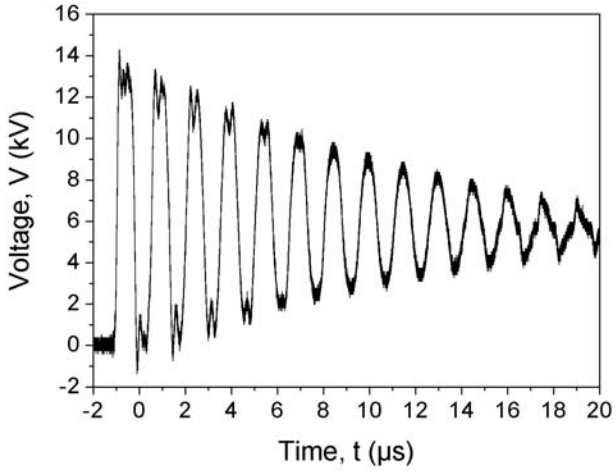

FIG. 3: Typical voltage waveform for the PEF system. The impedance of the Blumlein line pulse-forming network is much lower than the resistance of the PEF treatment chamber. Therefore, a train of 1 µs pulses with decreasing amplitudes is an applied instead of a single pulse.

TABLE 1: Summary of treatment parameters for standard extraction methods

Standard Method	Parameters	Treatment Time
Microwave	600 W	20 s
Ultrasound (sonication)	20 kHz	20 min
Homogenization	5000 PSI	8 min

TABLE 2: Summary of treatment parameters for plasma methods and PEF exposure

Plasma	Setup	Treatment Time
Corona discharges	Coaxial, directly in suspension; recirculation system	30 min
Plasma jet	DC jet; operated with air; submerged in suspension	30 min
VDBD	Above suspension (15mm)	10 min
Pin-to-liquid discharge	From pin to suspensions; distance 3 mm	3 min
Spark discharges	Rod-to-rod discharge (gap distance 0.5 mm) inside suspension; recirculation system	30 min
PEF	1 MS, 35 KV/CM, 5.5 HZ	1000 pulses

E. Measurement of Protein Content

To prove a successful rupture of the cell wall, the protein content in the supernatant was measured with the Bradford assay.[39] An increase of protein content can be used as a marker for efficient disintegration of microalgal cell walls. Therefore, after plasma treatment, the algae suspension was centrifuged at 3000 × *g* for 5 min. Then, 1 mL of supernatant was mixed with 1 mL of Bradford assay reagent (ThermoFisher Scientific, Germany) and measured at 595 nm with a UV/VIS spectrophotometer (Evolution 300, ThermoFischer Scientific, Madison, WI). Bovine standard serum albumin was used for calibration.

F. Pigment Extraction

Pigments such as chlorophylls and carotenoids are located inside chloroplasts, which are protected by the strong cell walls of microalgae. Therefore, an increase in concentration of these substances in the extract can be used as a marker for successful cell wall rupture. Extracted pigments were gained according to Luengo et al.[19,40] After centrifugation at 3000 × *g* for 5 min, the supernatant was discarded and 100 µL of treated or untreated algae (control) pellet suspension was mixed with 96% ethanol (V/V, Carl Roth, Germany) and thoroughly vortexed. Subsequently, the mixture was incubated in the dark for 20 min at room temperature and centrifuged for 2 min at 21 × *g* (Heraeus Pico21, ThermoScientific, Germany). The absorbance of the supernatant was measured at 450, 649, and 664 nm against a 96% ethanol blank. Calculations of total carotenoid, chlorophyll *a*, and chlorophyll *b* were conducted with the following equations:[41]

$$\text{Chlorophyll } a \text{ (Ca) in µg/mL: } (13.36 * A664) - (5.19 * A649)$$

$$\text{Chlorophyll } b \text{ (Cb) in mg/mL: } (27.43 * A649) - (8.12 * A664)$$

$$\text{Total carotenoids in µg/mL: } (1000 * A470 - 2.13 * Ca - 97.64 * Cb)/209$$

G. pH Measurements

Because acidification of the algae suspension may occur due to radical formation during plasma treatment, the pH was measured before and after plasma treatment and, for completeness, also for PEF treatment.[42] The liquid was analyzed with a pH meter (S-20 SevenEasy, Mettler Toledo, Giessen, Germany).

H. SEM

Damage to cell walls was also investigated directly by SEM (PhenomPro, PhenomWorld, Netherlands). For analysis, 10 µL of treated algae suspension was placed on a pin stub mount with a double-sided adhesive patch (PLANO GmbH, Wetzlar, Germany), dried with nitrogen, and transferred into the microscope.

I. Statistical Analysis

Experiments were done in triplicate if not stated otherwise. Means and standard deviations (SDs) of data were calculated. Error bars in all figures correspond to SD.

III. RESULTS

All algae samples had a volume of 50 mL with an OD (measured at 750 nm) of 0.8. Determination of extracted protein and pigment yields was acquired after treatment with standard methods, plasma, and PEF treatment, respectively, by the procedures as described in the "Measurement of protein content" and "Pigment extraction" sections. Experiments were conducted over a longer period of time, so different growth phases of algae affected the results. Pigment concentration in particular can vary for different growth phases. Accounting for differences in cultures and the effect of growth phases, algae cultures were analyzed individually; for each culture, 100 µL of algae suspension was extracted with 1 mL of 96% ethanol and extraction yields compared between microwave treatment results and their corresponding untreated control. Protein content was always determined with 1 mL of the supernatant, which corresponded to a total volume of 50 mL. Dry weight was determined to 0.1167 g/L (SD = 0.0287 g/L).

A. General Effects

In this work, the efficiency of three different standard extraction methods, five different plasma sources, and PEF treatment for rupturing algae cell wall are described. With the chosen model organism, a sufficient cell wall rupture could be achieved with microwave extraction and treatment with a spark discharge plasma source. In comparison, no satisfying rupture could be achieved with homogenization and ultrasound treatment. It could be shown that spark discharge treatment with a frequency of 4 Hz in a contentious flow system was as effective as microwave treatment or even better for pigment extraction. For all other plasma sources and PEF treatments, no effect or a smaller effect compared with microwave was observed.

B. Protein Extraction

The measurement of protein in the supernatant after treatment with different methods was chosen as a marker for successful cell wall rupture. Therefore, a protein assay was used. From the absorption at 595 nm, the relative protein concentration in the supernatant was determined. Results were related to dry weight (0.1167 g/L).

Figure 4 shows the protein content in the supernatant for the extraction by standard methods. With ultrasound, a protein content of 0.4% was achieved. Comparing homogenization and microwave treatment, microwave yielded more than twice the amount (5.9%) compared with homogenization (2.5%).

Protein extraction yield after plasma treatment or PEF treatment is shown in Fig. 5. Corona discharges, plasma jet, VDBD, pin-to-liquid discharge, and PEF achieved less

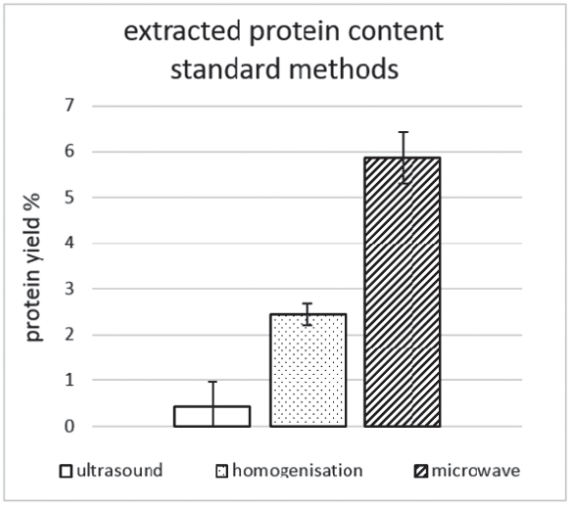

FIG. 4: Protein concentration (%) related to dry weight in supernatant after treatment with ultrasound, homogenization, and microwave. Each bar describes mean values from three independent experiments. Error bars indicate standard deviation (SD).

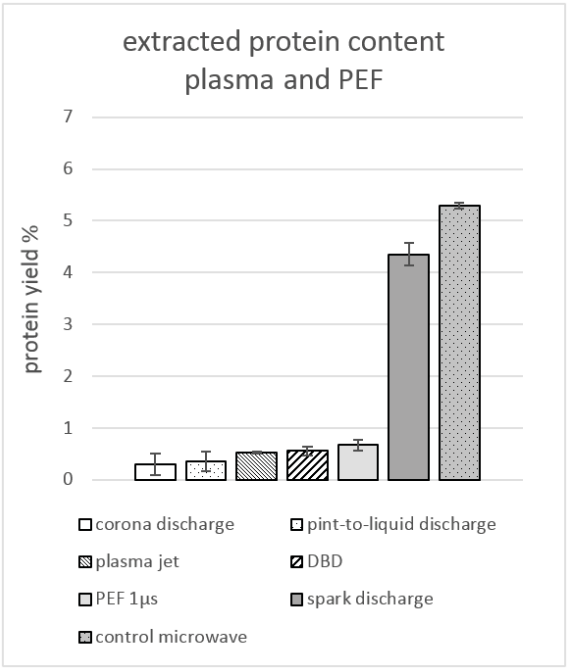

FIG. 5: Protein content (%) related to dry weight, in supernatant after plasma treatment with five different plasma sources and PEF. Each column represents mean values from three or 12 independent experiments (microwave n = 12). Error bars indicate SD.

than 1% protein in amount of the equivalent dry weight, whereas with the microwave standard, more than 5% protein was achieved. However, spark discharges are nearly as effective as microwave treatments. With this plasma source, operated at a spark frequency of 4 Hz, a protein yield of 4.35% was obtained.

C. Pigment Extraction

Pigments are located in the thylakoids inside the chloroplast, which is protected by the cell wall. An increase of chlorophyll *a* and *b* and total carotenoids in the extract therefore indicates successful cell wall rupture. In addition to the comparison with microwave extraction, results were also compared with an untreated control: a 100 µL *C. vulgaris* pellet suspension was extracted with ethanol 96% without any pretreatment. These experiments were conducted to estimate whether plasma had any effect at all due to growth-phase-dependent pigment content inside the algae. To account for variations due to individual cultures and growth phases, results for treatments are described with respect to this untreated control. All results are expressed as pigment concentration in mg/g culture$_{(dw)}$ (dw: dry weight). In figures, chlorophyll *a* is represented by "Chl a," chlorophyll *b* content by "Chl b," total carotenoid content by "TC."

1. Standard Methods

Results for the amount of extracted pigments after treatment with ultrasound, homogenization, or microwave exposure are shown in Fig. 6. As shown for the protein content, microwave extraction is the most effective technique in contrast to ultrasound and homogenization. The chlorophyll *a* content for ultrasound treatment is 1.317 mg/g culture$_{(dw)}$, for homogenization 1.935 mg/g culture$_{(dw)}$. An approximately 4- to 7-fold higher content was obtained with microwave treatment (8.613 mg/g culture$_{(dw)}$). Similar results can be shown for chlorophyll *b* and total carotenoid content. A yield of chlorophyll *b* of 2.680 mg/g culture$_{(dw)}$ and total carotenoid of 3.162 mg/g culture$_{(dw)}$ could be achieved. Only 0.686 mg/g culture$_{(dw)}$ for ultrasound and 0.855 mg/g culture$_{(dw)}$ for homogenization could be measured for chlorophyll *b*. In addition, only a small amount of carotenoids of 0.607 mg/g culture$_{(dw)}$ could be extracted with ultrasound and of 0.925 mg/g culture$_{(dw)}$ with homogenization.

2. Corona Discharges

Corona discharges were ignited directly in the algae suspension in a wire cylinder geometry. The results of pigment extraction are shown in Fig. 7. The amount of extracted pigments is increased compared with the untreated control. Nevertheless, the pigment amount extracted with microwaves is higher. In particular, the total carotenoid content is nearly four times higher (microwave 4.494 mg/g culture$_{(dw)}$, corona discharges 0.946 mg/g culture$_{(dw)}$). However, chlorophyll *b* content was 0.601 mg/g culture$_{(dw)}$: higher for corona discharges than microwave extraction. No positive effect for corona

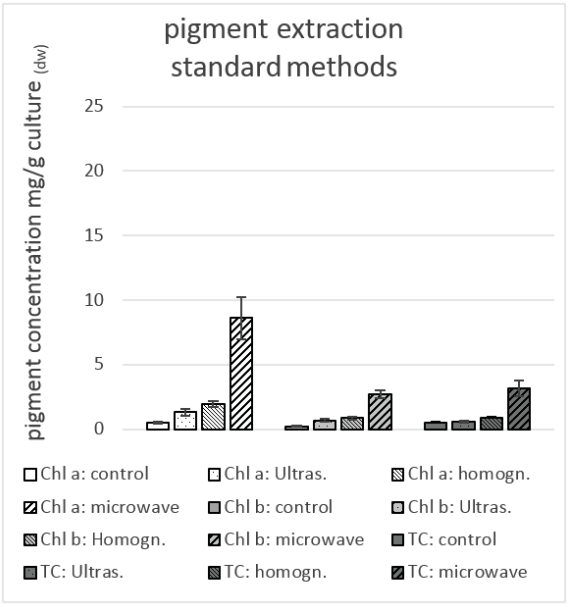

FIG. 6: Pigment concentration (mg/g culture$_{(dw)}$) after ultrasound (Ultras.), homogenization (homogn.), and microwave treatment. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

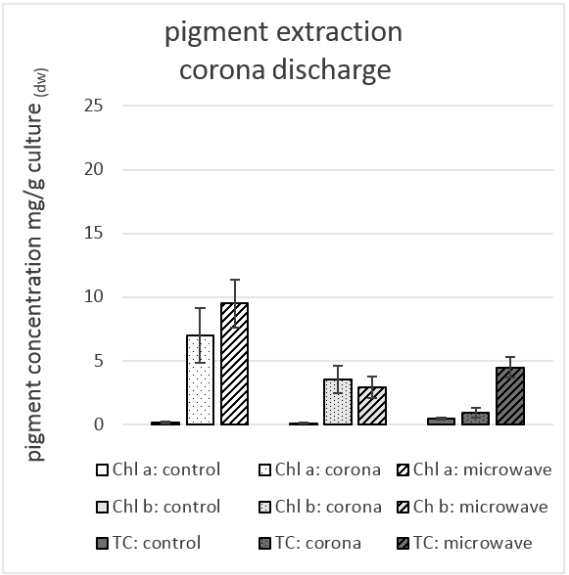

FIG. 7: Pigment concentration (mg/g culture$_{(dw)}$) after corona discharge treatment in a wire cylinder geometry as described in the "Corona discharges" section in the text. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

discharge treatment could be observed for extraction of chlorophyll *a*, either. The content for corona discharge was measured with 7.024 mg/g culture$_{(dw)}$. For microwave treatment an amount of 9.531 mg/g culture$_{(dw)}$ was determined.

3. Plasma Jet

The effect of plasma on pigment extraction generated with an air-driven plasma jet that was immersed in the treatment volume for 30 min was determined. Figure 8 shows the pigment yield after plasma jet treatment compared with microwave extraction and untreated control. Only a minor effect was observed when the algae suspension was treated with the plasma jet. Chlorophylls *a* and *b* extracted with microwave (15.350 mg/g culture$_{(dw)}$ and 4.566 mg/g culture$_{(dw)}$) were considerably higher than for the plasma jet-treated suspensions (0.842 mg/g culture$_{(dw)}$ and 0.210 mg/g culture$_{(dw)}$, respectively). A similar result could be shown for total carotenoid content extracted with microwaves and plasma jet (5.718 mg/g culture$_{(dw)}$ and 1.587 mg/g culture$_{(dw)}$, respectively).

4. Volume-DBDs

Microalgae suspensions were exposed for 10 min to the plasma of a VDBD and its effluents. The results (Fig. 9) show that this kind of plasma has no distinct effect on extracted pigment content compared with microwave extraction. The extracted amounts

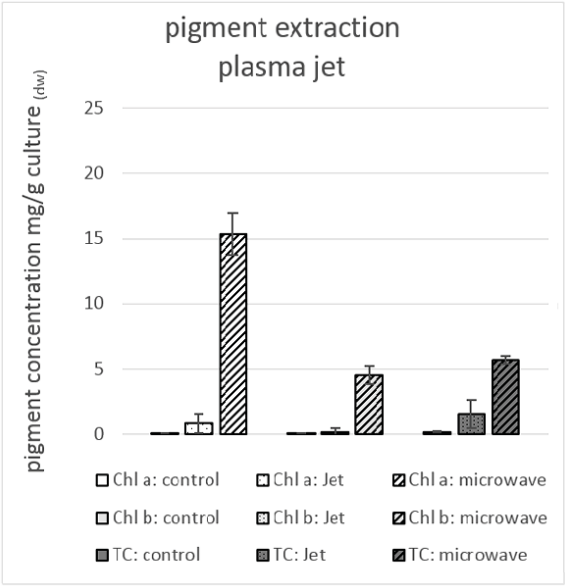

FIG. 8: Pigment concentration (mg/g culture$_{(dw)}$) after treatment with a plasma jet (Jet) as described in the "DC-plasma jet" section of the text. Each bar describes mean values of three independent experiments. Error bars indicate SD.

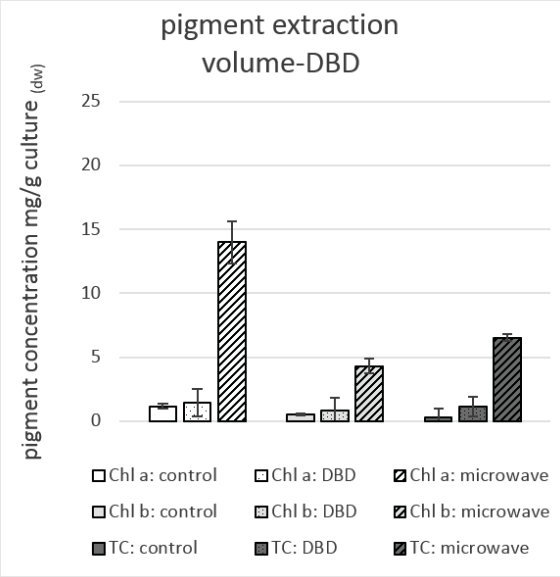

FIG. 9: Pigment concentration (mg/g culture$_{(dw)}$) after VDBD treatment as described in the "VDBD" section of the text. Each bar describes mean values from three and two independent experiments (n = 3, microwave n = 2). Error bars indicate SD.

after VDBD treatment are in a similar range as untreated control. Total carotenoid content after microwave treatment was 6.535 mg/g culture$_{(dw)}$ compared with 1.160 mg/g culture$_{(dw)}$ achieved after plasma treatment.

5. Pin-to-Liquid Discharge

In Fig. 10, the pigment concentration required after pin-to-liquid discharge treatment is shown. Contrary to the low protein content, the pigment concentration after this plasma treatment is increased compared with untreated control. However, the chlorophyll *a* and total carotenoid contents gained with the microwave method are still higher than those achieved with this plasma source. For microwave extraction, the total carotenoid content (3.069 mg/g culture$_{(dw)}$) is three times higher than pin-to-liquid discharge (1.021 mg/g culture$_{(dw)}$). Chlorophyll *a* is also 2 mg/g culture$_{(dw)}$ higher for the microwave treatment (6.836 mg/g culture$_{(dw)}$) than for the plasma treatment (4.809 mg/g culture$_{(dw)}$). Nevertheless, like corona discharges, the chlorophyll *b* content was increased after plasma treatment compared with microwave (3.124 mg/g culture$_{(dw)}$ and 2.17 mg/g culture$_{(dw)}$, respectively).

6. Spark Discharge

A remarkable rise in pigment concentration could be shown for a plasma treatment with spark discharges. The extracted pigment amounts are shown in Fig. 11. Nota-

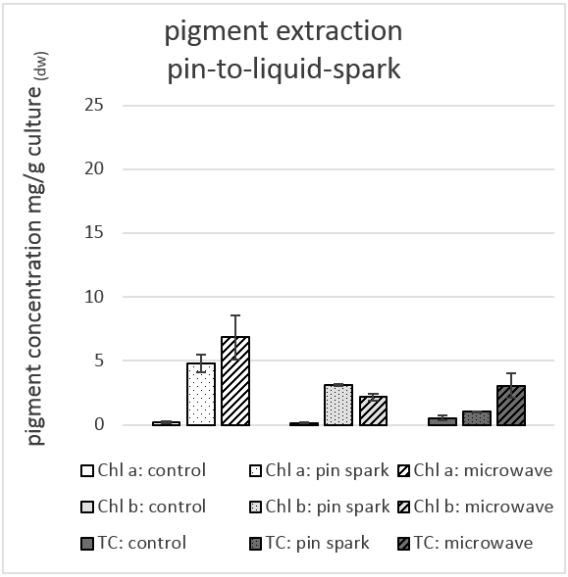

FIG. 10: Pigment concentration (mg/g culture$_{(dw)}$) after pin-to-liquid treatment (pin spark) as described in 2.4.4 of the text compared with microwave extraction and untreated control. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

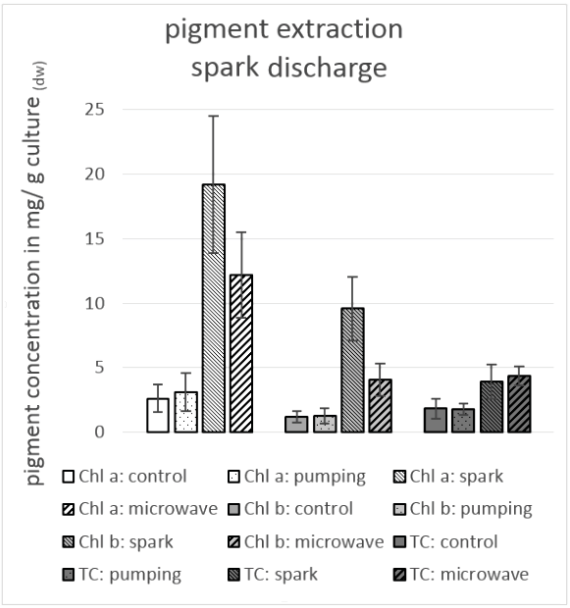

FIG. 11: Pigment concentration (mg/g culture$_{(dw)}$) after spark discharge treatment as described in the "Spark discharges" section of the text compared with microwave extraction. Each bar describes mean values from six or three independent experiments (n = 6, pumping alone n = 3). Error bars indicate SD.

ble, chlorophyll *a* content is increased 1.6-fold compared with microwave extraction (19.204 mg/g culture$_{(dw)}$ and 12.207 mg/g culture$_{(dw)}$, respectively). An increase in chlorophyll *b* can be also be observed for spark discharge treatment (9.583 mg/g culture$_{(dw)}$) compared with 4.058 mg/g culture$_{(dw)}$ for microwave. Furthermore, a comparable yield of total carotenoids after plasma treatment (3.952 mg/g culture$_{(dw)}$) was obtained compared with microwave extraction (4.386 mg/g culture$_{(dw)}$). To determine the influence of the continuous flow system, pigment extraction was conducted after 30 min of circulating the algae suspension through the system without applying any sparks. This did not result in an increased extraction yield compared to control, so algae cells seem to be unaffected by movement (results displayed as "pumping" in the diagram).

7. PEF Treatment

Pigment concentrations after PEF treatment were also determined and are shown in Fig. 12. Chlorophyll *a* and *b* extraction yields (0.280 mg/g culture$_{(dw)}$ and 0.249 mg/g culture$_{(dw)}$, respectively) were nearly the same as achieved for untreated control samples (0.249 mg/g culture$_{(dw)}$ and 0.275 mg/g culture$_{(dw)}$, respectively). A total carotenoid concentration of 0.726 mg/g culture$_{(dw)}$ for PEF-treated samples were obtained compared with the smaller amount for total carotenoid concentration of 0.468 mg/g culture$_{(dw)}$ for the untreated algae suspension. In contrast, after microwave treatment, a chlorophyll *a* content of 9.727 mg/g

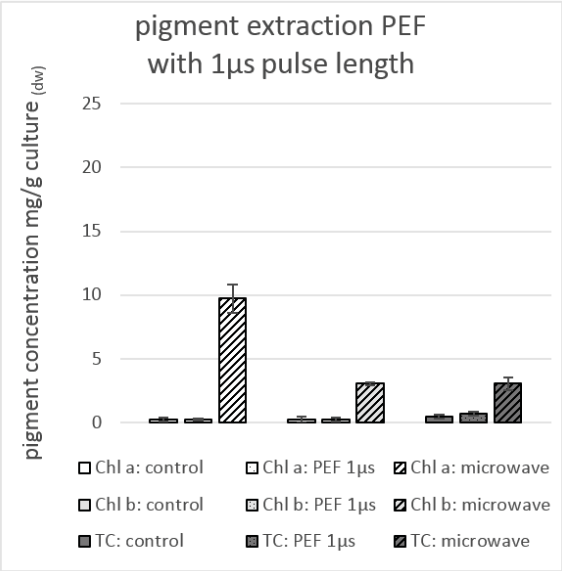

FIG. 12: Pigment concentration (mg/g culture $_{(dw)}$) after PEF treatment as described in the "PEF" section of the text. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

culture$_{(dw)}$ and a chlorophyll *b* content of 3.051 mg/g culture$_{(dw)}$ were obtained. A total carotenoid concentration of 3.871 mg/g culture$_{(dw)}$ could be measured as well.

D. Changes in pH Values

Plasmas have also been investigated with respect to the generation of reactive species that might have the potential to promote the rupture of cell walls, for example, through oxidative processes. Accordingly, a drop in pH values of the directly and indirectly treated aqueous suspensions can be observed. Individual experiments were therefore accompanied by measurements of pH values. Start values slightly differ depending on the respective microalgae culture. The end values for different plasma treatments and PEF exposures are presented in Table 3. Corona discharges and the plasma jet showed only a very slight effect on pH value: a drop from 6.00 to 5.05 for corona discharges and a small increase from 7.01 to 7.10 for the submerged plasma jet. In contrast, for the DBD and the spark discharge, a drop of 1.41–2.00 could be determined. With the pin-to-liquid discharge, the highest decrease in pH value was measured, indicating a strong reaction chemistry. A difference of 3.65 between the start pH value (6.73) and the end pH value (3.08) was achieved. In comparison, no significant change in pH value was detected for PEF treatment; only a minor decrease from 7.50 to 7.30 was observed.

TABLE 3: pH measurements before and after plasma and PEF treatments

	Corona Discharge	Plasma Jet	VDBD	Spark Discharge	Pin-to-Liquid	PEF
Start	6.00	7.01	6.07	6.77	6.73	7.50
End	5.50	7.10	4.66	4.77	3.08	7.30

E. SEM images

To verify potential cell damage by different methods, SEM images were taken. Images for microwave treatment, spark discharge treatment, and untreated algae are shown in Fig. 13. Usually, *C. vulgaris* has a round shape and a smooth surface, which can be seen in Fig. 13*a*. If an algae suspension is exposed to microwave radiation, a single hole on the cell surface can be observed (Fig. 13*b*). In comparison, the spark discharge treated algae appear to be wizened and some are deflated completely (Fig. 13*c*). In both cases, the cell wall is apparently ruptured; however, different mechanisms seem to be responsible. All other tested methods (i.e., homogenization, ultrasound, corona discharges, plasma jet, VDBD, pin-to-liquid discharges, and PEF) showed no differences between untreated control and treated samples (respective images are therefore not shown).

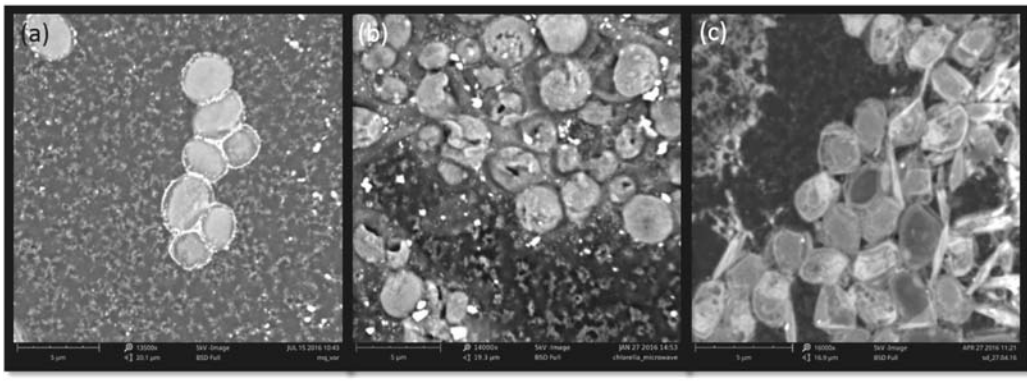

FIG. 13: SEM images of C. vulgaris before any treatment (a), after microwave treatment (b), and after spark discharge treatment (c).

IV. DISCUSSION

The objective of this work was a comparison of selected standard extraction methods, novel plasma methods, and PEF with respect to improving extraction yields of valuable compounds from microalgae. Therefore, five different plasma sources and PEF treatment were chosen and compared with treatments with ultrasound, homogenization, and microwave exposure. Different physical and chemical reaction pathways of plasmas have been exploited to different degrees by the different methods.

For the applied standard methods, the mechanisms of cell wall disruption are understood. When cells are treated with ultrasound waves, cavitation phenomena occur, leading to cell rupture. During this cavitation process, microbubbles are formed, which expand in the rarefaction phase of the sound wave. Subsequently, the bubbles are jolted in the compression phase, resulting in a collapse of these microbubbles. Collapsed microbubbles release a shock wave that propagates throughout the liquid; therefore, a high amount of sonic energy is converted into mechanical energy.[43] A disadvantage of the method is the associated considerable temperature increase due to cavitation. Therefore, chilling of the suspension is necessary to prevent heating and destruction of components.[8,44] We could observe that sonication of *C. vulgaris* has only a minor effect on cell disruption, supposedly due to its remarkable strong, rigid cell wall. No major increase in extracted protein content and pigment concentration could be determined. The effect of sonication and homogenization on cell disintegration for lipid extraction from *C. vulgaris* was investigated previously by Park et al.[12] For conditions similar to our experiments, a frequency of 20 kHz and 20 min treatment time, they could likewise not observe significant cell wall rupture. In addition, they could not find a significant effect for homogenization. According to Park et al., it is likely that cavitation bubbles and induced shockwaves, which occur under sonication processes, fail to disrupt the three thick and strong layers of the cell wall for this type of algae.[12] This assessment was

confirmed by Lee et al., who tested different disruption methods on *C. vulgaris*. Sonication with a frequency of 10 kHz and a treatment time of 5 min had only a minor effect on the disruption of microalgae.[45]

The underlying mechanisms of homogenization of biological cells are strong shear forces. The cell suspension of a certain volume is compressed in a small chamber and pressed through a narrow gap, resulting in cavitation processes. The sudden increase of energy in a small volume results in strong shear stress and, therefore, cell disintegration is possible.[3] In agreement with the results of Park et al., a sufficient cell wall rupture with homogenization could not be achieved in our experiments. Only a low increase of protein content in the supernatant and of pigment concentration was determined. It is likely that, not only the rigid cell walls of *C. vulgaris*, but also its small round shape is responsible for insufficient cell disintegration. Probably, the round shape of these microalgae enables it to resist high pressure changes. However, higher pressures might be more effective.

A strong increase of protein content in the supernatant and pigment concentration was achieved with microwave extraction. When comparing different cell rupture techniques, Lee et al. found that microwave extraction seems to be most suitable to break the cell walls of *C. vulgaris*. They could show that the extracted lipid content was highest (10% w/w) compared with sonication, bead-beating, autoclaving, and osmotic shock.[45] Microwave exposure causes migration of ions and rotation of dipoles. These molecular motions result in a rapid heating of the algae suspensions, particularly of water molecules inside the cells. The temperature increase results in a fast expansion of cells until the wall is ruptured and the cell content is released into the adjacent solvent.[46, 47] This might explain the single hole that was observed on the cell wall surface in the SEM images. Apparently, water inside the cells has been heated rapidly. With the associated increase in pressure, cell and cell wall are eventually ruptured in a weak spot, releasing content through the hole, thus effectively bursting open from the inside. It should be pointed out that the rather high temperatures related to microwave treatment can simultaneously have an adverse effect on sensible compounds such as proteins, chlorophylls, and carotenoids. Accordingly, a loss in activity of these compounds may occur. The harmful temperature effects of microwave exposure in our experiments can be seen in Fig. 14. The color of the pigment extract was yellowish-green compared with the lush green color gained with spark discharges and the light green of untreated control extract.

Standard extraction methods in general are usually based on mechanical damage, fast heating, or chemical reactions such as oxidation. The disadvantages of these methods include insufficient cell wall disintegration, long treatment times, a need for harsh solvents, or thermal degradation of compounds. With the investigated plasma exposures, it might be able to avoid or at least alleviate these shortcomings by exploiting other mechanisms. Plasmas have been shown to provide mechanical forces and oxidizing chemical species such as hydroxyl radicals, H_2O_2, or peroxynitrite.[23,42,48,49] Depending on the specific type of plasma source, different pathways and reaction chemistries are available.[31,32,42,50,51] Therefore, five different plasma sources generating different types of plasmas and thus different

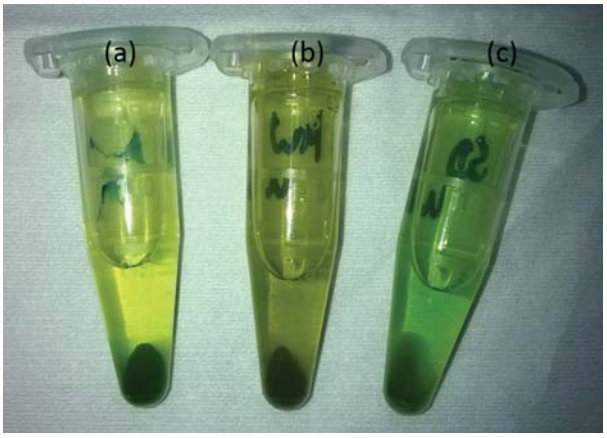

FIG. 14: Color images of extracted pigments for untreated control (a), after microwave exposure (b), and after spark discharge treatment (c). The dark objects at the bottom of the tubes are the centrifuged algae pellets.

physical and chemical characteristics were tested to determine which effect may be the reason for successful cell wall rupture. A common indication for the different processes is changes in pH values. The specific reaction chemistry for all plasma sources has been described thoroughly elsewhere.[31,32,52–55]

A decrease of pH values after a 10 min treatment time was found for VDBD to indicate the generation of chemical reactive species such as reactive nitrogen species, which recombine to nitric acid, leading to liquid acidification. In addition, the generation of H_2O_2 due to reactive oxygen species can be found for such a DBD system.[51,55] In contrast, plasma generated by the investigated jet had no influence on pH values, which stayed constant during the treatment time. However, from previous studies, it is known that in particular peroxynitrites are formed in aqueous solutions.[28] However, for both methods, the exposure of the cells to shear forces seems to be unlikely. Altogether, the experiments show that the chemistry provided by either VDBD or plasma jet are not sufficient to affect the cell wall. This has also been confirmed by SEM images (data not shown).

No drop of pH value was also measured for the application of corona discharges. A detailed study conducted by Banaschik et al. has shown that copious amounts of OH radicals and subsequently H_2O_2 are generated in this system.[31] The provided OH reaction chemistry does not seem to have an effect on the cell wall of *C. vulgaris*. In particular, because the total carotenoid content in the supernatant was lower than with microwave extraction and cell wall rupture was not detected, the generated shockwaves in the system do also not seem to be strong enough for the disintegration of *C. vulgaris*. That chemistry and shockwaves have no effect on cell walls can be seen in the SEM images (data not shown). This (in comparison) higher extraction yield could be attributed to PEF. In a previous work, we could show that a very strong electric field is established in the close vicinity of the wire electrode at the center of the reactor with and without the generation of corona

discharges. A calculated field strength directly around the wire (of 50 μm in diameter) of approximately 2.7 MV/cm was achieved for an applied voltage of 80 kV.[56] Therefore, a cell wall rupture could not be achieved, but a pore formation due to the electric field might occur. This electroporation might further explain the low protein content of the supernatant, but an increase of chlorophyll concentration compared with microwave extraction. Luengo et al. could demonstrate that pulses in the microsecond and millisecond range can increase pigment concentration compared with untreated microalgae. For example, a field strength of 20 kV/cm for a pulse length of 3 μs and a treatment time of 75 μs resulted in an increase of extraction yields for carotenoids and chlorophylls (*a* and *b*) of 42%, 54%, and 195%, respectively, when extraction was conducted after 1 h of preincubation. It was also described that no significant effect could be observed when extraction was conducted immediately after PEF treatment. Luengo et al. suggested that, because pigments are located in thylakoids inside the chloroplast, these compounds first have to cross the chloroplast membrane and afterward the cell membrane.[19,40] The treatment time in this work was set to 30 min, so a retarded chloroplast membrane poration may facilitate compound transport outside the cell.

The possibility of increasing the extraction yield from *C. vulgaris* by electroporation has motivated the experiments with PEF alone. The exposure showed no change in pH value because the generation of oxidizing species was not expected and only physically induced damage is expected. In this work, no effect on the cell wall by the applied PEF could be detected, as can be seen from the low protein content in the supernatant. The results are in agreement with previous observations by Postma et al. In their work, it could be shown that permeabilization of algal cell wall due to PEF treatment was not effective enough to release high quantities of large molecules such as proteins.[57] SEM images showed no influence of PEF on cell wall structure either, which confirms previous findings for the cell walls of bacteria.[21] Moreover, the pigment concentration stayed in the same range as for the untreated control, supporting that a cell wall destruction due to PEF treatment, as was also discussed by Günerken et al., seems unlikely.[3] Taking into account overall volume and continuous flow rate of the setup used in this work, about 58 pulse trains with a pulse length of individual pulses of 1 μs and a field strength of up to 35 kV/cm were applied to each algae cell while circulating through the treatment chamber during the treatment time. For the application of 50 pulses with a pulse length of 3 μs and a maximum field strength of 25 kV/cm, an increased pigment yield was observed by Luengo et al.[19] However, for our treatment conditions, we found no significantly higher concentrations. In addition to the longer pulse durations used by Luengo et al., which should, however, be compensated in part by the application of pulse trains in our case, differences might also be due to differences in experimental procedures. Pigment extraction was conducted immediately after PEF treatment as for plasma experiments. However, as Luengo et al. and also Goettel et al. suggest in their work, 1–2 h between PEF application and extraction might be necessary to achieve efficient yields of compounds from algae.[19,38] However, neither these experiments nor ours suggest a rupturing of cell walls.

The highest change of pH values was measured for the pin-to-liquid discharge, indicating a strong reaction chemistry. Before the plasma was switched on, the algae suspension had a pH value of 6.73, but after treatment, the pH value was 3.08. The formation of high amounts of nitrogen radicals such as peroxynitrites and other nitrogen oxides are the main reason for the drop of pH values.[42] However, the formation of these radical species seem not to be effective in rupturing cell walls either, because only a low protein content was measured after treatment. In addition, the SEM images showed no ruptured cells. Few cells appear dented slightly, but not ruptured. Interestingly, the chlorophyll content in the supernatant similar to that after the microwave treatment was observed. Again, the reason might be the electroporation of the cell membrane underneath the cell wall. Bruggeman et al. determined electric fields of 49.5 kV/cm in a comparable configuration, although operated by a DC voltage.[58] The curvature of our electrodes was even smaller, so the electric fields were higher. At least near the electrodes, an efficient electroporation process could be established, even for the AC voltage operation of the discharge. At an operating frequency of 50 Hz, the respective pulse duration given by half waves and pulse numbers within 3 min of treatment time are expected to be sufficient. A considerable disadvantage of the method, regardless, is the temperature increase in the suspension, which will degrade heat-sensitive pigments and other compounds.

The highest extraction yields with respect to both proteins and carotenoids were obtained for spark discharge treatments. Responsible for the results could be the reaction chemistry, but were more likely the strong and fast shockwaves from the rapid high-voltage breakdown for the short applied high voltage pulses. Before plasma application, the suspension had a pH value of 6.77 and after treatment a pH value of 4. Although the radical chemistry for our system has not been determined yet, a wide range of radicals seem to be generated. Comparable pH values were reported by Sugiarto et al., when the decoloration of dyes by pulsed discharges in water were investigated.[59] The formation of radicals in a plasma system depends on whether the spark or arc discharges are generated directly in water or if the high voltage electrode is placed above the water. Spark discharges in water have shown to produce OH, H, and O radicals and H_2O_2.[53,60,61] Therefore, produced species are similar to the once observed for corona discharges generated in water. However, Sun et al. found that spark discharges produce even higher amounts of OH radicals than streamer discharges due to a higher plasma density, although in a smaller volume.[62] Regardless, the similarities in reaction chemistries of spark discharge treatments and corona discharge treatments, together with the lack of enhanced extraction yields observed for the latter, are a strong indication that oxidation processes are not responsible for the results observed for the spark discharge treatment. In summary, an active influence of radicals generated by plasma on disintegration of cell walls seems unlikely.

Corona discharges and spark discharges are also known for the formation of shockwaves, providing another conceivable mechanism responsible for cell wall rupture. Sugiarto and Sato have investigated the degradation of phenol with streamer and spark discharges and concluded that spark discharges are more effective in particular for a

small treatment volume. In their work, they argue that this is due to a single strong plasma channel in the spark discharge, whereas the energy is dissipated among multiple paths in a corona discharge. Accordingly, a much stronger shockwave is formed during the propagation of the plasma channel. In this plasma channel, radical formation could also be enhanced.[63] Locke et al. describe in their work that streamer corona discharges form relatively weak shock waves compared with spark discharges.[61] Bousetta et al. and Rajha et al. observed that shockwaves are most likely the major mechanism for the structural damage to vine roots and grape seeds that are treated by pulsed arcs. As a result, polyphenol content is higher compared with PEF treatment or streamer discharge exposure.[16,18]

Because spark plasmas are associated with high temperatures, it is necessary to consider possible temperature effects that are associated with the treatment. In this work, a spark repetition rate of 4 Hz was found, a reasonable compromise with respect to cell wall rupture at moderate temperature increase. In this case, temperature could be held at around 25°C without any chilling system. The suspension maintained a lush green color compared with the thermal damage by microwave treatment, which resulted in similar extraction yields (Fig. 14). Decreasing the repetition rate of spark discharges to 2 Hz (for the same treatment time), protein content in the supernatant was half the one achieved with microwave treatment. Conversely, a frequency of 7 Hz resulted in a higher protein yield than microwave exposures, but temperatures rose above 32°C and algae suspension changed its dark green color to greyish-green (data not shown), indicating thermal degradation and thus loss of valuable components.

In general, spark discharges with a suitable frequency seem to be efficient for cell wall rupture of microalgae, yet are a gentle method to extract thermosensitive compounds such as pigments. Whether formed radicals have an influence on the extracted components in any way will have to be investigated in more detail. Further studies also need to focus on electrode geometries and the generation process of shockwaves by high-voltage pulses of different duration and amplitude.

V. CONCLUSION

In this work, the efficiency of three different standard extraction methods, including microwave treatment, five different plasma treatments, and PEF exposure for rupturing the cell walls of *C. vulgaris* are described. It could be shown that microwave extraction was best suitable for comparison with highest extraction yields. However, disadvantages are very high temperatures and a low scalability. Valuable, but heat-sensitive compounds may be destroyed and an economic large-scale implementation is difficult. Plasma sources, providing strong shock waves, were found to be a potential alternative. Plasma sources with only strong reaction chemistries proved to have a very small effect on cell walls, resulting in small extraction yields. Likewise, PEF alone are inefficient. Spark discharges applied with a frequency of 4 Hz achieved extraction yields similar to microwave exposures, but without thermal degradation of compounds. Details of mechanisms, effect on extracted compounds, and energy demand have to be investigated in future studies.

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